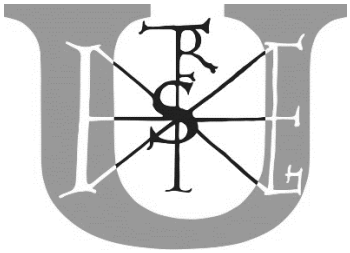
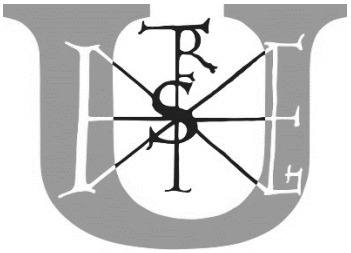


Szent István University

Thesis for Doctoral degree (Ph.D.)

Lázár Bence
Gödöllő
2020



Szent István University

ISOLATION AND CHARACTERISATION OF AVIAN PRIMORDIAL GERM CELLS *IN VITRO* AND *IN VIVO*

Lázár Bence
Gödöllő
2020

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1. INTRODUCTION AND AIMS

1.1. Introduction

Over the last decade, the European Union has strongly supported the establishment of gene banks and the storage and use of gene bank samples. Since the 1950s, native Hungarian poultry and waterfowl species and breeds have been kept (Biszkup and Beke, 1951; Báldy, 1954) as living gene bank populations (*ex situ in vivo*). To coordinate the use of genetic material in gene banks within the European Union and to improve techniques (*ex situ in vitro* methods), we set out to create primordial germ cell (PGC) gene bank samples besides the already stored Hungarian indigenous poultry semen samples. In avian species, PGC storage in gene banks has great importance because recently this has become the most practical way to preserve the genetic material present in the female W chromosome and mitochondrial DNA.

In birds, unlike mammals, the male has the homogametic ZZ chromosomes, while the female has the heterogamous ZW chromosome pair. Since only Z haploid genetic material can be conserved in sperm, 6-8-steps of backcrossing is required to reconstruct the original genome (Blesbois, 2007).

The use of stem cells in birds for breed constitution began in the 1990s (Tajima et al., 1993; Naito et al., 1994). However, to take advantage of their potential, a method was needed to propagate PGCs *in vitro* to increase their number. Van de Lavoir *et al* (Van De Lavoir et al., 2006) first developed a long-term culturing method, and later on, further improvements had been made by other researchers (Macdonald et al., 2010; Whyte et al., 2015; Miyahara et al., 2016; Tonus et al., 2016). Nowadays, PG cells can be readily obtained from the blood of avian embryos (48-65 hours after laying in chicken), they can be cultivated, deep-frozen, and then re-injected into the circulation system of a recipient embryo. Thus, they can be used for gene conservation, research, and genetic modification purposes.

As there are many research papers on this topic (Pain et al., 1996; Nakamura et al., 2010; Macdonald et al., 2010; Kim et al., 2013; Rikimaru et al., 2014; Whyte et al., 2015; Tonus et al., 2016), nonetheless there is no established practice and widely accepted standard protocol on the production and validation of PGC lines to be stored in gene banks, in this study a methodology is proposed for this purpose.

1.2. Aims

- To adapt methods for isolation, cell line establishment, *in vitro* culture, and freezing of PGCs from indigenous Hungarian chicken breeds.
- To establish a PGC-based gene bank that contains samples from six indigenous Hungarian chicken breeds.
- To verify the sample quality and the effectiveness of the applied methods: generation of Patridge Colour Hungarian germline chimeras and donor-derived offspring from PGC samples stored in the gene bank.
- To develop a standard protocol for generating good quality samples for PGC-based gene-banking of chicken breeds.

2. METHODS

2.1. Characteristics of the species and varieties involved in the experiments

2.1.1. Hungarian chicken breeds

The progenitors of these birds were brought into the Carpathian Basin from Asia by the Hungarian conquerors at the end of the ninth century. Over the centuries of their formation, these breeds have become well adapted to the special climatic conditions and farming systems of the country, which made them very precious local varieties in the Carpathian Basin (Szalay, 2015). The following breeds were used during the investigations of this study: Yellow Hungarian, White Hungarian, Speckled Hungarian, and Partridge Coloured Hungarian.

2.1.2. Transylvanian Naked Neck chicken breeds

The neck, partly the breast, and the belly of Transylvanian Naked Neck chickens are characteristically featherless. The naked neck trait is controlled by an autosomal gene and dominant in relation to the feathered neck trait. In the case of homozygous naked neck birds, the neck is completely naked, whereas heterozygous birds have a feather brush on the front, the lower part of their neck, which can already be observed on the day-old chicks. There is only a little plumage on the top of their head. They are extraordinarily hardy, firm and resistant, grow and feather fast, perform good egg production and the weight of the eggs can exceed 70g, however, they have a weak brooding instinct (Szalay, 2015). More color variations can be distinguished; Black Transylvanian Naked Neck and the Speckled Transylvanian Naked Neck were used during these experiments.

2.2. Ethics approval

Animals were kept and maintained according to general animal welfare prescriptions of the Hungarian Animal Protection Law (1998; XXVIII). All experimental methods described herein were approved by the Institutional Ethics Review Board of the Institute for Farm Animal Gene Conservation (No. 7/2011).

2.3. Maintenance of domestic fowl experimental stocks

The fertile eggs which were used for establishing primordial germ cell lines came from the gene bank stock of the National Centre for Biodiversity and Gene Conservation, Institute for Farm Animal Gene Conservation. The old Hungarian chicken breeds were kept in barns with large outdoor areas in the institute. The stocking density 5-6 birds/m^2, the sex ratio: 7 hens, 1 cockerel. There are nest boxes (5 hens/nest) for the collection of eggs. Breeding flocks are fed with laying mash in addition to limestone grit. The eggs are collected twice a day, and then stored in a refrigerated room. The hatched chickens were grown in a special chick-rearing box until 4 weeks of age (0.5 m^2/10 individuals). The box is equipped with

automatic heating and lighting. The temperature was gradually reduced weekly from 30°C to the final 22-24°C. After 4 weeks of age, they were placed on deep litter. The young individuals were fed *ad libitum* with granulated starter mash (Szinbád Ltd., Gödöllő, Hungary) and a water supply (Molnár et al., 2019).

2.4. Isolation of PGCs from the embryos

Blood was collected individually from every embryo between HH stages 13-17 (2.5-3 days old). 1-3 µL blood was isolated from the dorsal aorta under a stereomicroscope, using sterile pulled glass microcapillary (~ 20 µm in diameter at the end) and a mouth pipette, then immediately placed into 48-well plates, in selective media for chicken PGCs (Whyte et al., 2015). Tissue samples for sex-determination were collected from every isolated embryo and stored at -20°C until further use. The time of isolation, the exact age of the embryos (HH stages), and the presence/absence of developmental abnormalities were recorded.

2.5. Establishment and maintenance of the PGC lines

The isolated blood from single embryos, which contains the cellular components of blood including the PGCs, was cultured *in vitro* in selective media for PGCs (Whyte et al., 2015), in a thermostat at 38°C with 5% of CO_2 concentration for 3 weeks. This period of culturing ensures enough time for the elimination of all cell types except the PGCs. In the selective medium, PGCs start to divide, and a homogeneous cell population can be established. If the cell number of PGCs from one embryo reached 1.0×10^5 cells in 3 weeks, the establishment of the line was considered successful.

2.6. *In vitro* characterization of the PGC lines

The established PGC lines were submitted to *in vitro* validation in order to define the essential characteristics and ensure that the cell populations are homogenous and have the specific characteristics of this cell type.

2.6.1. DNA Isolation and Sex Determination

For isolating the DNA, High Pure PCR Template Preparation Kit (Roche Diagnostics, US) was used according to the manufacturer's instruction. The sex of the donor embryos and the established PGC lines were determined with the P2–P8 primer set as described before by Griffiths and colleagues (Griffiths et al., 1998), or with the CHD1 primer set published by Lee and colleagues (Lee et al., 2010). The isolated DNA was diluted to 25 ng/µL concentration for PCR reaction and gel electrophoresis. MyTaq Red Mix was used for the reaction (Bioline Reagents Ltd., UK). The PCR products were then separated by electrophoresis, using 1.5% agarose gel stained with ethidium bromide at 90V for 30 minutes. The DNA bands were then visualized under UV illumination and photographed (Lázár et al., 2018).

2.6.2. Immunostaining of PGCs

Isolated PGCs were fixed with 4% PFA for 10 minutes. After washing with PBS (three times, five minutes each), cells were permeabilized with 0.5% Triton X-100 (Merck Millipore, US) for 5 minutes. After washing with PBS, to minimize nonspecific binding of antibodies, the fixed cells were blocked for 45 minutes with a blocking buffer containing PBS with 1% (v/v) BSA. Cells were then washed three times with PBS and were incubated with each of the primary antibodies. After incubation overnight in the primary antibody solution in a humid chamber at 4°C, the cells were washed three times with PBS. Then, cells were incubated with the secondary antibodies, in a dark humid chamber for 1 hour at room temperature. After washing with PBS, the nucleus was stained with TO-PRO-3 stain (1:500, T3605, Molecular Probes Inc., US), which is a far-red fluorescent (642/661) nuclear and chromosome counterstain. Coverslips were mounted on the slide with the application of 20µl VECTASHIELD Mounting Media (H-1000, Vector Laboratories Inc., US) and analyzed by confocal microscopy (TCS SP8, Leica) (Lázár et al., 2018).

2.6.3. Isolation of RNA, Synthesis of cDNA, and Quantitative Real-Time PCR

Total RNA from the established PGC lines was isolated using RNAqueous-Micro Total RNA Isolation Kit (Thermo Fisher Scientific, MA, USA) following the instructions of the manufacturer. The concentration of RNA was determined by NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA), and the samples were stored at −70°C until later use. The extracted RNA samples were reverse transcribed into cDNA with High Capacity cDNA Reverse Transcription Kit following the instructions of the manufacturer (Applied Biosystems, Life Technologies, Carlsbad, US). RT Master Mix was used for cDNA writing. The cDNA was stored at −20°C. The synthesized cDNA was then used for quantitative real-time PCR. SYBR Green PCR Master Mix was applied for the qPCR as a double-stranded fluorescent DNA-specific dye according to the manufacturer's instructions (Applied Biosystems, Life Technologies, Carlsbad, US). For each gene examined, triplicates were measured, fluorescence emission was detected, and relative quantification was calculated with the GenEx software (MultiD, SE) (Lázár et al., 2018).

2.6.4. Cell Proliferation Assay

Cell lines were analyzed for the rate of proliferation. Proliferation was measured with the CCK-8 reagent (Sigma-Aldrich, 96992). The strength of the CCK-8 reaction (bright orange color) is directly proportional to live cell number in the culture, therefore data was collected by measuring absorbance (optical density: O.D.) at 450 nm using a CLARIOstar® Microplate Reader (BMG Labtech, US). PGC lines from the same breed were individually measured in the same experiment. The measurements were taken over three days, once per day. Triplicate wells were measured every day for every cell line on a 96-well plate (Lázár et al., 2018). Average absorbance values from each day were used to calculate doubling time (DT) for each cell line with the following formula: $DT = d * \log(2) / \log(A_t) - \log(A_0)$, where d: duration in days; A_t: absorbance at the third day; A_0: absorbance at the first day (Kong et al., 2018).

2.6.5. Microsatellite marker analysis

17 microsatellite markers were used in the experiments which were selected based on an earlier project (AVIANDIV, https://aviandiv.fli.de/). The detection of the PCR products was performed with an automated DNA sequencer (GenomeLab™ GeXP Genetic Analysis System, Beckman Coulter, CA, USA), according to the instructions of the manufacturer. For determining the allele sizes, a 400 bp long ladder was used. The collected data was analyzed with the GenomeLab Genetic Analysis System software (Beckman Coulter, Inc. 4300 North Harbor Boulevard, Fullerton, CA 92834-3100). The inbreeding coefficients (F_{IS}) were determined and compared with the FSTAT software (Goudet, 2001).

2.7. *In vivo* characterization of the PGC lines

The cultured PGC lines were characterized in vivo as well. To check the functionality and migration ability of the PGC lines, fluorescently labeled cells were injected into recipient embryos.

2.7.1. Fluorescent labeling of frozen/thawed cells

Cultured PGCs were marked with a vital cell surface fluorescent dye, which renders the injected cells visible in the embryo. PGCs were centrifuged and after washing in sterile PBS, the red (PKH26 Red Fluorescent Cell Linker, Sigma Aldrich, MIDI26-1KT) or green (PKH67 Green Fluorescent Cell Linker, Sigma Aldrich, MIDI67-1KT) dye was applied according to the manufacturer's description. Fluorescence of freshly labeled cells was checked with a confocal microscope (TCS SP8, Leica), then the cells were put into CO_2 incubator until subsequent use (Lázár et al., 2018).

2.7.2. Assessing the capability of PGC migration into the gonad

Fluorescently labeled cells were collected and counted (Arthur Cell Analyzer, NanoEnTek Inc., Seoul, Korea). After the centrifugation, the pellet was resuspended in a mixture of DMEM and sterile water with a ratio 2:1 (simplified media was used, because some components of the complete PGC media may affect the development of embryos). The recipient embryos were from the same breed. After the shell of recipient eggs were cleaned with alcohol, a hole was opened with a diameter of 1 cm, and then 1 µL of the prepared cell suspension (~3-5000 PG cells/µL) was injected into the heart of 2.5-day-old embryos using a pulled glass microcapillary. After the injection, ~50 µL of preheated sterile 1xPBS was dropped on to the embryo, then the hole was closed with 2 layers of sterile laboratory parafilm and the individually marked eggs were put back into the hatchery.

2.7.3. Monitoring of integration of injected cells into the gonads

After the injection, the eggs were incubated for four days. Embryos were isolated and the gonads were dissected from the embryos. The gonads were washed in sterile 1x PBS, then fixed in 4% PFA overnight. The injected cells were identified in the fixed gonads with a fluorescence stereomicroscope and photo documentation was taken.

2.8. Freezing of PGC lines

Freshly prepared freezing media for PGCs was used for freezing of the established PGC lines, which contained the following components: DMEM (Gibco, 21068-028) and sterile water (Gibco, 15230-089) in a 2:1 ratio, 8% of DMSO (Sigma Aldrich, 276855), 10% of chicken serum (Sigma Aldrich, C5405) and 0.75% of 20 mM CaCl2 (Sigma Aldrich, C4901). PGCs were suspended carefully from the bottom of the culturing well and then pipetted into 1.5 mL Eppendorf tubes. After centrifugation (1000 x g, 3 minutes) the supernatant was removed, then the cells were suspended in 250 μL of DMSO free freezing media for PGCs and pipetted into labeled cryotube. 250 μL of freezing media for PGCs was added slowly dropwise, and then the tube was placed into the freezer at -70°C. In the case of long-term storage, after one night, the samples were moved into the freezer at -150°C or into liquid nitrogen.

2.9. Thawing of PGC lines

For thawing, of PGCs, a water bath at 37°C was used, and then the total content of the tube was pipetted into 2 mL of culturing media for PGCs. After centrifugation (1000 x g, 3 minutes) the supernatant was removed, then the cells were resuspended in fresh culture media for PGCs and placed into cell culture wells for further growth.

2.10. Establishing the gene bank with PGC lines

Individual PGC lines established from every breed were initially kept in 48-well plates then transferred to 24-well plates. Once the cells filled out the wells, they were halved and distributed into two wells. This process was repeated until there were 6 parallel wells from each cell lines. The cell mass generated in such way was finally mixed and then distributed into 6 freezing tubes. The freezing protocol was applied as described in chapter 2.8. Three of the tubes were transferred to NBGK HGI and are stored in the gene bank of the institute in liquid nitrogen; the other three tubes are in the gene bank of NAIK MBK and stored at -150°C. The separation of the samples was a safety measure in case of unexpected events. Cell suspension samples were also collected and stored at -150°C for subsequent DNA and RNA isolation.

2.11. Regenerating the donor breed with injection and crossing

For this experiment, I aimed to prove that the *in vitro* cultured and frozen PGC lines are capable to create germline chimeras and with crossing the chimeras donor-derived offspring.

Partridge Coloured Hungarian chicken was chosen as a donor breed, because the partridge color is a recessive trait; it is expressed only if both parents are pure partridge colored. In the recipient breed, the Black Transylvanian Naked Neck chicken, the gene for naked neck trait is dominant, thus if partridge colored covered neck chickens hatch after the backcrossing, they are derived from injected PGCs.

2.11.1. Injection of donor-derived PGCs into recipient embryos

PGCs were cultured as detailed above. PGCs were frozen and then thawed before injection. A male and a female cell line (No. PC101 and PC111 lines) were selected for injection (Lázár et al., 2018). Recipient eggs were incubated until stage HH13-17. 3000-5000 mixed-sex PGCs were injected into the heart of each embryo through a hole (1 cm in diameter) on the eggshell. After the injection, 50 µL of sterile 1x D-PBS was added and then 2 layers of parafilm were used to close the hole. The injected eggs were incubated at 37.8°C with 70% relative humidity.

2.11.2. Incubation of recipient embryos and examination of the embryonic development

Incubation of the recipient eggs was done in a Midi F500S incubator at 37.8°C and 60-65 % relative humidity. After the injection, eggs were put back into the incubator until hatching. The development of the embryos was monitored with egg candling after day 10. In case of the developmental abnormality or embryonic death, the potential cause was documented.

2.11.3. Test crossings of the presumptive germline chimeras

The presumptive chimera Black Transylvanian Naked Neck chickens were placed into individual cages for mating. For test crossing, Partridge Coloured Hungarian mates were used as they were the donor breed, and were also kept in individual cages. Two Partridge Coloured Hungarian hens were inseminated artificially with the sperm of each Black Transylvanian Naked Neck presumptive chimera cockerel. The Black Transylvanian Naked Neck presumptive chimera hens were inseminated with mixed sperm of 5 Partridge Coloured Hungarian cockerels.

2.11.4. Artificial insemination

Semen from cockerels was collected by abdominal massage according to Burrows and Quinn (Burrows and Quinn, 1935). The fresh, pooled and diluted semen was inseminated in a dose of 100±20 million spermatozoa per female in all cases. For the calculation of sperm concentration, Lake's diluent was used. Artificial insemination of hens was performed as described by Bakst and Dymond (Bakst and Dymond, 2013).

2.12. Statistical analysis

The following software was used to analyze the data: Excel (MS Office), RStudio (Version 1.3.959), R (R-4.0.2), GeneEx (6.0), and Microsatellite Toolkit (Park, 2002). Levels of significance were applied as follows: $*p<005$, $**p<001$, and $***p<0001$. In the case of every qPCR run, expression changes of the target genes were calculated compared to the level of the housekeeping gene with the standard $2^{(-\Delta\Delta Ct)}$ method, where Ct = cycle threshold; ΔCt = Ct (target gene) − Ct (housekeeping gene) and $\Delta\Delta Ct = \Delta Ct$ (test sample) − ΔCt (control sample). In case of the doubling time (DT) data, Mann-Whitney tests were used to compare the groups.

3. RESULTS

3.1. Isolation and establishment of stable PGC lines

Blood and PGCs were isolated from six Hungarian native breeds and we managed to establish robust cell lines from all 6 breeds. Altogether 396 isolations were performed and 166 cell line establishments were done which is a 42.0% average derivation rate. It was around 50% for 3 Hungarian breeds (Yellow, White, and Speckled Hungarian), which were the most successful. Among the Hungarian varieties, Partridge Colour proved to be the worst; the derivation rate was only 28%. The derivation rate of two Transylvanian Naked Neck breeds was slightly below 40%. In the case of every breed, at least 20 individual cell lines were created. Six parallel cryotubes from each cell line were placed in the gene bank, every vial containing approximately $5.0x10^4$ cells (Table 1.). The number of isolations for each breed depended on egg availability, egg fertility, and the number of healthy embryos in the right developmental stage.

Table 1. Derivation rates of established cell lines and the number of stored samples in the gene bank.

Hungarian indigenous breeds	No. of isolations	No. of cell cultures	No. of male cell lines (%)	No. of female cell lines (%)	Derivation rate (%)	No. of cryotubes in genebank
Yellow Hungarian	80	40	29 (72.5)	11 (27.5)	50.0	240
White Hungarian	42	20	14 (70.0)	6 (30.0)	47.6	120
Speckled Hungarian	76	37	27 (73.0)	10 (27.0)	48.7	222
Partridge colour	74	21	17 (81.0)	4 (19.0)	28.4	126
Black Transylvanian Naked Neck	68	27	23 (85.2)	4 (14.8)	39.7	162
Speckled Transylvanian Naked Neck	56	21	11 (52.4)	10 (47.6)	37.5	126
Σ (%)	396	166 (42.0)	121 (72.3)	45 (27.7)	-	996

3.2. *In vitro* characterization of the PGC lines

3.2.1. Sex determination

Sex determination was performed from embryonic tissue samples collected after the isolation. Out of 166 stable PGC lines, there were 121 (72.3%) male and 45 (27.7%) female cell lines. The theoretical 50-50% sex ratio was not met in any of the cases. The Speckled

Transylvanian Naked Neck was the closest with 47.6% of the cell lines being female. Though the culturing media is capable of maintaining both male and female cell lines, further improvement is needed to match the requirement of the female cell lines better.

3.2.2. Immunohistochemistry analysis

Immunohistochemistry of PGCs was performed on cell lines of all six breeds. Two cell lines, one male and one female were selected randomly from each breed. Cells were collected after culturing and freeze-thawing the cell lines. Each of the tested cell lines showed germ cell-specific CVH/DAZL and stem cell-specific SSEA1 staining. CVH and DAZL are cytoplasmic while SSEA1 is a cell surface marker. TO-PRO-3 was used as a nuclear stain.

3.2.3. Gene expression analysis

PGC lines were analyzed from four breeds for marker expression analysis using qPCR. Two male and two female cell lines were chosen randomly from every chicken breed. All tested cell lines showed expression of the germ cell-specific *CVH* and the stem cell-specific *cPOUV* markers. The housekeeping gene was the *cGAPDH* in all of the cases. All PGC lines highly expressed the stem and germ cell-specific markers. Chicken embryonic fibroblast (CEF) cells were used as a negative control (Figure 1.).

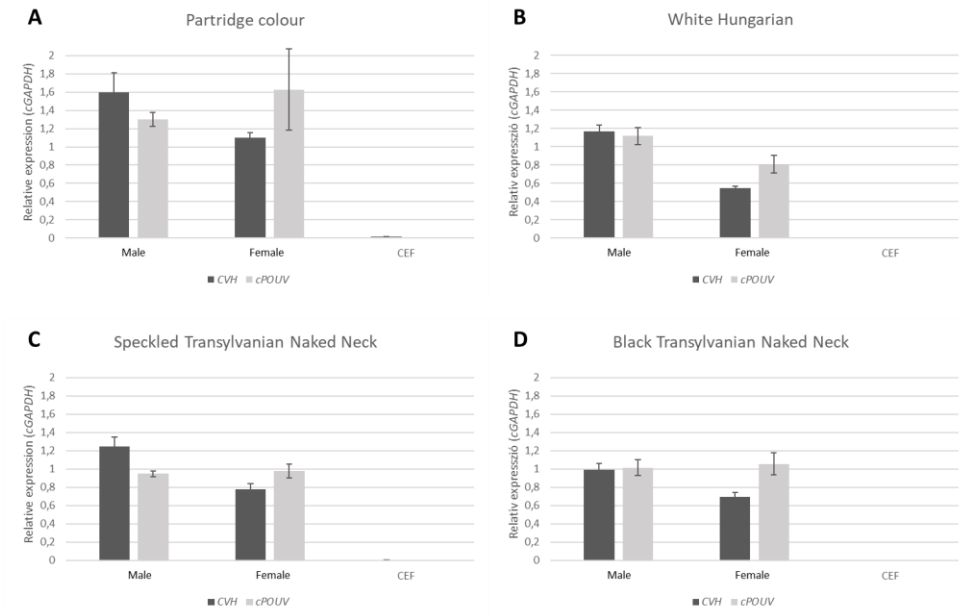

Figure 1. Germ cell and stem cell-specific marker expression of PGC lines. Expression levels of *CVH* and *cPOUV* genes compared to the *cGAPDH* housekeeping gene were high in all the examined PGC lines in all breeds. Expression levels from chicken embryonic fibroblast (CEF) cells are present as a negative control. **A:** Partridge Coloured Hungarian, **B:** White Hungarian, **C:** Speckled Transylvanian Naked Neck, **D:** Black Transylvanian Naked Neck.

3.2.4. Cell division analysis

We calculated the doubling time (days) for different PGC lines. In general, all the analyzed PGC cultures showed appropriate proliferation, which means relatively shorter doubling times, therefore they were considered as valuable lines for gene banking purposes. The speed of cell proliferation is an important factor for cell lines established for gene banking purposes. Cell lines with relatively fast proliferation (shorter doubling time) are easier and quicker to work with, but on the other hand, the genetic value is not necessarily linked to proliferation. Since the main purpose was to preserve as much genetic diversity as possible, we did not set a strict doubling time threshold for the indigenous PGC lines. If any given cell line could produce enough cells for the *in vitro* experiments and gene bank samples, it was cryopreserved. Analyzing the data from all of the breeds I did not find a significant difference between the male and female lines, but if the sexes were examined in each breed separately significant difference was found in 2 cases (Partridge Coloured Hungarian and Speckled Hungarian) (Figure 3.). Then I compared the breeds and found that out of 10 comparisons there is a significant difference between 7 breed pairs (Figure 2.).

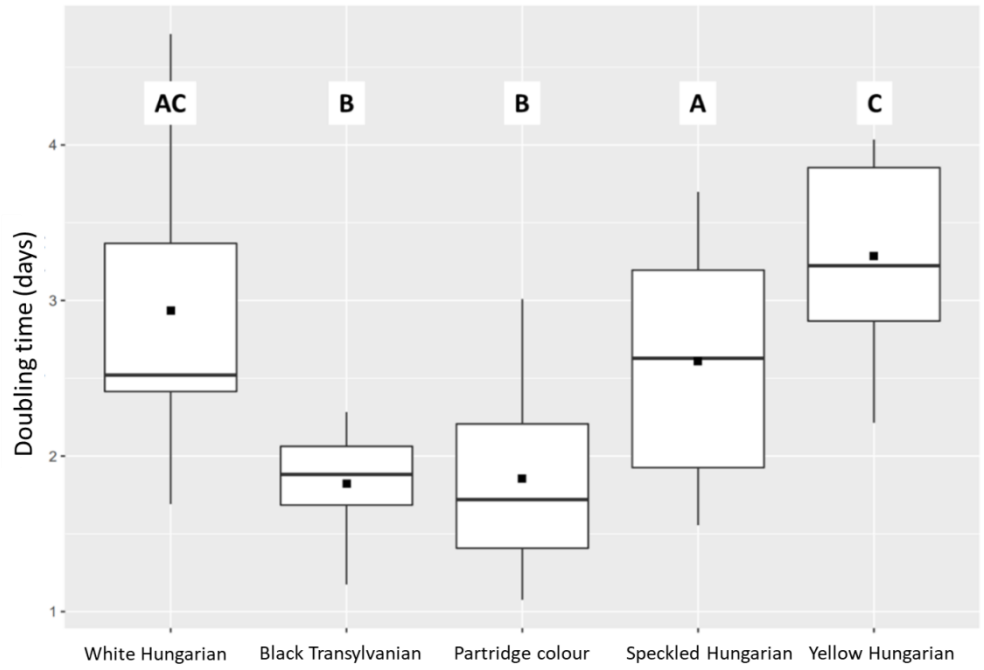

Figure 2. Doubling time (DT) data showed for five Hungarian breeds. I compared the breeds and found that out of 10 comparisons there is a significant difference between 7 breed pairs. Mann-Whitney tests were performed, different letters indicate a significant difference between breeds. On the boxplots, black dots represent the mean values and black lines indicate the median values.

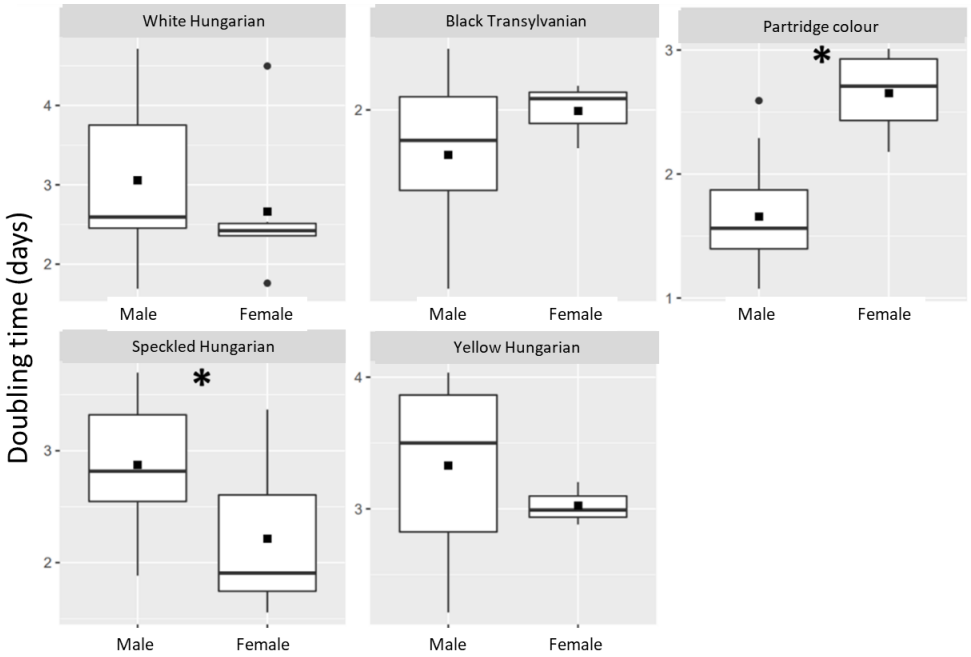

Figure 3. Doubling time (DT) data showed for male and female PGC lines of five Hungarian breeds. Analyzing all of the data from the five breeds, I did not find a significant difference between the male and female lines, but if the sexes were examined separately for each breed, a significant difference was found in 2 cases (Partridge Colour and Speckled Hungarian). Mann-Whitney tests were performed, significant differences are marked with an asterisk. On the boxplots, black dots represent the mean values and black lines indicate the median values.

3.2.5. Microsatellite marker analysis

In this experiment, my aim was to determine whether the 21 Partridge Coloured Hungarian PGC lines are a good representation of the flock held in the NGBK HGI. 30 samples of the NBGK HGI population were used from a previous study to answer this question with 17 microsatellite markers. General diversity parameters such as mean number of alleles, allele frequency, expected and observed heterozygosity were calculated (Table 2.). Based on these results, the established PGC lines are a good representation of the flock in the NBGK HGI. Nevertheless, it is important to mention that there were markers that were present only in one of the populations. This can be because of the relatively small sample size, and with the inclusion of more samples from both populations probably these markers could have been found. A significant difference was found between the expected and observed heterozygosity of the control group, but in the case of the PGC lines, no difference was present.

Table 2. Results of the microsatellite marker analysis. (MNA: mean number of alleles, H_E: expected heterozygosity, H_O: observed heterozygosity, SD: standard deviation, F_{IS}: inbreeding coefficient (***P<0.001))

Population	MNA±SD	H_E±SD	H_O±SD	F_{IS}
Established PGC lines	3,59±2,00	0,52±0,05	0,50±0,03	0,04
Control samples	3,76±1,44	0,52±0,05	0,43±0,02	0,18***

3.3. *In vivo* characterization of the PGC lines

3.3.1. Validation of PGC lines by injecting fluorescently labeled cells

The cultured PGCs were characterized *in vivo* to investigate the cell function, and their ability to migrate into the gonads. An *in vivo* colonization test was done on PGCs from five Hungarian chicken breeds. In total 118 injections were performed and 69 embryos (58.5%) survived the procedure. In the case of 60 embryos, donor-derived PGCs were found in the gonads which is an 87.0% average colonization rate (Table 3.). In the Yellow Hungarian, White Hungarian, and Black Transylvanian Naked Neck breeds, fluorescently labeled donor cells were found in 100% of the surviving embryos. The Partridge Coloured Hungarian and the Speckled Transylvanian Naked Neck varieties showed colonization rates between 76-78%.

Table 3. Results of the *in vivo* colonization test of five Hungarian indigenous chicken breeds

Hungarian indigenous breeds	No. of injections	No. of live embryos (%)	No. of colonizations	Rate of chimeras (%)
Yellow Hungarian	**20**	**13 (65,0%)**	**13**	**100,0%**
White Hungarian	**15**	**8 (53,3%)**	**8**	**100,0%**
Partridge Coloured Hungarian	**32**	**21 (65,6%)**	**16**	**76,2%**
Black Transylvanian Naked Neck	**19**	**9 (47,4%)**	**9**	**100,0%**
Speckled Transylvanian Naked Neck	**32**	**18 (56,3%)**	**14**	**77,8%**
Sum (%)	**118**	**69 (58,5%)**	**60 (87,0%)**	**-**

3.4. Establishing the gene bank with PGC lines

Individual PGC lines established from every breed were initially kept in 48-well plates then transferred to 24-well plates. In the case of every established cell line, 6 x (5 x 104) cells in total were frozen. Three of the tubes were transferred to NBGK HGI and are stored in the gene bank of the institute in liquid nitrogen; the other three tubes are in the gene bank of NAIK MBK and stored at -150°C. The separation of the samples was a safety measure in case of unexpected events. Cell suspension samples were also collected and stored at -150°C for subsequent DNA and RNA isolation. 972 freezing tubes were stored in total for the six breeds (Table 1.)

3.5. Recovering the donor breed with injection and crossing

3.5.1. Injection of donor PGCs

From five experiments, 52 Black Transylvanian Naked Neck Chicken embryos were injected as recipients on the 2.5th day of incubation with frozen/thawed PG cells of Partridge Coloured Hungarian chicken. A mixture of a male (No. PC101) and a female (No. PC111) Partridge Coloured PG cell lines in ratio 1:1 were used for injection. From 52 injected eggs, 29 chicks (55.8%) were hatched and 24 of them (82.8%) were raised until sexual maturity. 13 males and 11 females were produced.

3.5.2. Test-cross of the presumptive germline chimeras

During the mating experiments, 795 eggs were incubated over 9 weeks. 340 chicks were hatched and 14 of them (4.1%) were pure partridge colored with a covered neck. Based on the mating results 4 out of 24 presumptive chimeras (16.6%) were proven to be germline chimeras: 1 hen and 3 roosters. Therefore, I have proven that the original breed can be recovered from both male and female PGCs which are stored in the gene bank.

3.6. Proposed protocol for PGC-based gene-banking of chicken genetic resources

Along with establishing a PGC-based gene bank for the Hungarian indigenous chicken breeds, we aimed to propose a general protocol for efficiently producing and storing high-quality PGC lines to meet preservation goals. After reviewing the established methods and considering our own experiences, we propose a simple protocol for culturing and quality control of PGC lines. After completing these steps, it will be safe to assume that the cell lines match the *in vitro* and *in vivo* characteristics of a high-quality PGC line and they will be useful for future regeneration of the breed/species. The proposed steps and criteria to be met are summarized in Table 4. Further information about the techniques can be found in the Methods section.

Table 4. A proposed general protocol for establishing and testing PGC lines for gene banking purposes.

	Steps	Criteria
1	Isolation of blood from donor embryos	Min. 50 isolations
2	Establishing PGC cultures in feeder-free conditions	Min. 1,0 x 10^5 PGCs in 3 weeks / cell line
3	Sex determination of cell lines	Min. 4-6 female cell lines
5	Immunohistochemistry of selected cell lines	CVH / DAZL and SSEA-1 positive cells
6	Gene expression analysis of selected cell lines	*CVH and cPOUV* expression
7	*In vivo* migration assay with fluorescently labeled cell lines	Integration into the gonads of recipients
8	Freezing of established and tested cell lines	Min. 6 vials, min. 5,0 x 10^4 cells/vial

3.7. New Scientific achievements

1. I established and *in vitro* cultured male and female PGC lines from six indigenous Hungarian chicken breeds (White Hungarian, Yellow Hungarian, Partridge Coloured Hungarian, Speckled Hungarian, Black Transylvanian Naked Neck, Speckled Transylvanian Naked Neck); furthermore, I established a PGC-based gene bank which contains samples from all six breeds.
2. I created germline chimeras with PGC lines from five indigenous Hungarian breeds (White Hungarian, Yellow Hungarian, Partridge Coloured Hungarian, Black Transylvanian Naked Neck, Speckled Transylvanian Naked Neck).
3. I successfully recovered the donor breed with PGC samples from the gene bank in the case of the Partridge Coloured Hungarian breed.
4. I proposed a general protocol for establishing and testing PGC lines for gene banking purposes in chicken.

4. DISCUSSION

There are 3689 avian breed populations recorded worldwide (of which 2222 are indigenous breeds) and 28% of these breeds are endangered, vulnerable, or already extinct (FAO, DAD-IS, http://www.fao.org/3/CA0121EN/ca0121en.pdf). For the ~8800 recorded breeds of 40 domesticated animal species, 7% of the breeds are already extinct and 24% are at risk of extinction (FAO 2019, http://www.fao.org/resources/infographics/ infographics-details/en/c/174199/). Looking at this data, it is evident that wild birds and native poultry breeds alike are at great risk. Preservation of the genetic resources in local chicken breeds not only helps to maintain the biodiversity in poultry but also provides valuable material that we might seek and use in crossbreeding strategies in the future (Wang et al., 2017).

The alteration in the diverse use of PGCs occurred after 2006 when it became possible to proliferate and maintain PGCs in cell culture (Van De Lavoir et al., 2012). However, in the "Cryoconservation of animal genetic resources" Guidelines published by the FAO in 2012 (FAO 2012), gene conservation with PGCs still exists only at a theoretical level. FAO did not include any protocols that could be used for that purpose. Therefore, in this study, our aim was not only to present our results on establishing the first Hungarian PGC-based gene bank but also to propose a uniformly recommended protocol for the isolation, establishment, cryopreservation, and testing of PGC lines used for gene banking in chicken. To date, several research groups have already succeeded in recovering indigenous chicken breeds from PGCs (Nakamura et al., 2010; Rikimaru et al., 2011; Tonus et al., 2016; Wang et al., 2017; Yu et al., 2019) but most of them did not use cell lines or if cell lines were involved they propagated them with feeder cells and/or only male cell lines were established. To our knowledge, only Woodcock and colleagues used stable PGC lines from both sexes derived from a specialized breed and applied feeder-free culturing conditions (Woodcock et al., 2019).

Our study is unique in how we used feeder-free culturing conditions for both sexes and also using multiple indigenous breeds that cover most of the Hungarian local chicken breed spectrum. The advantage of the *in vitro* culturing method we have adapted (Whyte et al., 2015) to the Hungarian indigenous breeds is that female cell lines of all breeds could be established and maintained in this selective medium and succeeded in obtaining female germline chimeras and donor-derived offspring. A further advantage of the recommended cell line establishment and maintenance protocol is that it is easy to apply as it does not use feeder cells.

In the current study, we successfully isolated PGCs from six Hungarian indigenous breeds and maintained both sexes *in vitro*. The average establishment rate of the PGC lines (42.0%) is similar to other studies based on specialized breeds and we obtained more male PGC lines than female lines (Nandi et al., 2016; Woodcock et al., 2019). The derived PGC lines were positive for SSEA-1, and CVH, and expressed the germ-cell specific genes *CVH* and *cPOUV*. Donor-derived offspring were produced with 4.1% efficiency which is comparable to the results presented in previous experiments (Nakamura et al., 2010, 2013; Miyahara et al., 2014; Yu et al., 2019). The *in vitro* characterization and *in vivo* validation of PGC lines described above can be used to ensure that PGC samples placed in the gene bank will be suitable for recovering the endangered, rare, or native breeds. Also, this proves that the germ cell lines established by this method and placed in the gene bank are suitable for the recovery of the original breed.

4.1. Recommendations

In the case of the chicken, all the components of a successful *in vitro* strategy are present: isolation, *in vitro* culture, freezing, and quality control techniques are all well established. The key component of the gene preservation process is the cell culture medium. The one which is adequate for chicken cells, not necessarily working with other species, therefore the future of this research area will contain quite a lot of medium development. Giving the right elements for the cells for the proper division is not easy, the signaling pathways of PGCs from other species must be examined, and with that data new recipes could be developed. Thus, in my opininon, this is one of the topics researchers of this area should focus on. The other key aspect of genetic preservation with PGCs is the competition in the recipient which is present because of the endogenous PGCs of the embryo. Therefore it would be really important to create universal recipients with decreased PGC number and a wide range of applicability for other species. This would improve the efficiency of regenerating the donor breed greatly and open the way for wild bird species to undergo PGC-based conservation projects. In summary, I recommend continuing to broaden the list of breeds and species we are able to preserve with PGC-based gene banking, and I encourage the researchers of related fields to further improve our understanding on medium development and potential recipient animals/concepts. With this approach, the now *in situ* and *ex situ in vivo* protected breeds and even wild species could be much more resilient to the future.

5. SCIENTIFIC PUBLICATIONS - SELECTION

Journal articles with an impact factor related to the topic of the thesis:

- **Lázár, B.**, Molnár, M., Sztán, N., Végi, B., Drobnyák, Á., Tóth, R., Tokodyné Szabadi, N., McGrew, M. J., Gócza, E., Patakiné Várkonyi, E. (2020): Successful cryopreservation and regeneration of a Partridge coloured Hungarian native chicken breed using primordial germ cells. **POULTRY SCIENCE** *under review*.
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- Tóth Roland, **Lázár Bence**, Tokodyné Szabadi Nikolett, Patakiné Várkonyi Eszter, Gócza Elen (2019): Őshonos magyar tyúkfajták, mint lehetséges univerzális recipiensek az ősivarsejt alapú génmegőrzésben. **MAGYAR ÁLLATORVOSOK LAPJA** 141/439-447. **Q4, IF: 0,143**
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- Patakiné Várkonyi, E., Gócza, E., **Lázár, B**., Sztán, N, (2017): **Mindkét ivar megőrzése embrionális sejtek segítségével különböző baromfifajokban.** In: Szalay István (szerk.) Génbanki kutatások régi haszonállataink védelmében: Műhelytanulmányok a tudományos génmegőrzés tárgyköréből. 214p. Budapest; Gödöllő: Haszonállat-génmegőrzési Központ, 2017, pp.113-122. (ISBN 978-963-286-729-8)

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- **Lázár Bence** (2018): Őshonos házityúk fajtáink génmegőrzése ősivarsejtek felhasználásával. ***GÉNNET-21, „A Kárpát-medencei őshonos haszonállatfajok, -fajták és -ökotípusok XXI. századi génbanki stratégiájának tudományos megalapozása és fejlesztése***", szakmai tanácskozás, Gödöllő, HáGK, 2018. június 7., magyar nyelvű előadás
- **Lázár, B**., Tóth, R., Nagy, A., Anand, M., Liptói, K., Patakiné Várkonyi, E., Gócza, E. (2017): Primordial germ cell-based biobanking of Hungarian indigenous chicken breeds. Poultry Science 96:(E Suppl.1.) p62 (IF: 1.908). ***Poultry Science Association 106th Annual Meeting***, Orlando, USA, 2017.07.17 -2017.07.20. (World' s Poultry Science Association) OP-166, absztrakt, előadás (Lázár Bence)
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- **Lázár, B.**, Südy, Á., Németh, K., Bontovics, B., Gócza, E. (2015): Examination the expression of pluripotency related genes in the gonads and primordial germ cells of the chicken. ***EMBO Workshop: Embryonic – Extraembryonic Interfaces***, Göttingen, 6-9 May 2015, poszter, absztrakt, rövid előadás (Lázár Bence)

Poster presentations related to the topic of the thesis:

- **Lázár Bence**, Tóth Roland, Tokodyné Szabadi Nikolett, Molnár Mariann, Patakiné Várkonyi Eszter, Gócza Elen (2019): Ősivarsejtek *in vitro* jellemzése és embrionális lokalizációjának vizsgálata lúdban (*Anser anser domestica*). ***25. Szaporodásbiológiai Találkozó***, 2019.11.08-09, Balatonkenese, poszter (Lázár Bence)
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